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Pharmacological evaluation of the neurotoxic components of *Parabuthus Transvaalicus* scorpion venom for potential analgesic development

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Abstract
Chronic pain, a global health burden exacerbated by the limitations and risks of current opioid-based therapies, necessitates the discovery of novel, non-addictive analgesics. Animal venoms represent rich, evolutionarily refined libraries of ion channel modulators with high potential for drug development. The venom of the medically significant scorpion *Parabuthus transvaalicus* is a potent neurotoxic cocktail, primarily causing excruciating pain, yet paradoxically it may harbour compounds capable of silencing nociception. This study provides a comprehensive pharmacological evaluation of *P. transvaalicus* venom to isolate and characterize neurotoxic components for their analgesic potential.
Sequential chromatographic fractionation of the crude venom yielded 32 discrete fractions, from which eight principal neurotoxins (Pt2, Pt5, Pt8, Pt11, Pt15, Pt22, Pt25, and Pt28) were purified and characterized. Automated and manual patch-clamp electrophysiology on recombinant human ion channels revealed starkly divergent pharmacological profiles. While known toxins like the β-Na_V toxin Pt2 acted as a broad-spectrum sodium channel activator (shifting V_{1/2} of activation by -15 to -25 mV), the novel long-chain toxin Pt25 emerged as a potent and selective inhibitor of the key pain-related isoforms Na_V1.7 (IC₅₀ = 8.7 ± 1.2 nM) and Na_V1.8, displaying >50-fold selectivity over cardiac Na_V1.5. Furthermore, the novel short-chain toxin Pt22 was identified as a positive allosteric modulator of K_V7.2/7.3 channels (M-current), causing a -18.5-mV shift in the voltage dependence of activation (EC₅₀ = 88 nM), a novel mechanism for a scorpion venom peptide.
In vivo behavioural assays in murine models confirmed this functional duality. Local injection of Pt2 induced immediate pain and hyperalgesia, whereas systemic administration of Pt25 (1 µg/kg, s.c.) or Pt22 (10 µg/kg, s.c.) produced significant analgesia in both acute nociceptive and chronic constriction injury (CCI) neuropathic pain models, without impairing motor function. Notably, sub-effective doses of Pt25 and Pt22 demonstrated synergistic analgesic interaction. Rational *in silico* design and mutagenesis yielded an optimized analogue of Pt25 (Pt25-M1) with enhanced selectivity and an improved therapeutic index.
This work successfully deconvolutes the dual nature of *P. transvaalicus* venom, transitioning it from a source of pathology to a viable resource for analgesic discovery. We identify Pt25 as a highly selective Na_V1.7 inhibitor lead and Pt22 as a first-in-class scorpion venom-derived K_V7 potentiator, providing two promising, mechanistically distinct templates for the development of next-generation, non-opioid pain therapeutics.

Keywords: *Parabuthus transvaalicus*, scorpion venom, neurotoxin, analgesia, sodium channel, chronic pain, venom peptide, drug discovery

Introduction

1. The Global Pain Crisis and the Venom me as a Therapeutic Reservoir

Chronic pain represents one of the most pervasive and debilitating global health challenges, affecting an estimated 20% of the world's population and imposing enormous socioeconomic burdens. Current mainstay analgesics, particularly opioids, are fraught with significant limitations, including tolerance, addiction, respiratory depression, and inadequate efficacy against neuropathic pain states. The ongoing opioid epidemic underscores the critical and unmet need for novel, non-addictive analgesic agents that target alternative molecular pathways. In this landscape, biodiverse natural venoms—evolved over millions of years to precisely disrupt the physiological homeostasis of prey and predators—have re-emerged as invaluable repositories of highly specific ion channel modulators, offering unprecedented templates for next-generation therapeutics [1].
Scorpion venoms, in particular, constitute complex chemical arsenals comprising salts, nucleotides, enzymes, protease inhibitors, and a dominant pharmacopeia of disulfide-bridged neurotoxic peptides. These peptides, typically 20–80 amino acids in length, exhibit exquisite selectivity and high potency for a range of voltage-gated and ligand-gated ion channels that govern neuronal excitability, synaptic transmission, and, crucially, nociceptive signalling. This inherent bioactivity has positioned scorpion toxins as powerful molecular probes for dissecting pain pathways and, more recently, as lead compounds for analgesic drug development. Notable examples include the chlorotoxin-inspired imaging agents and the ongoing clinical investigation of certain peptides for autoimmune diseases,

yet their potential for pain management remains largely underexploited [2].

Among the >2,000 scorpion species, those of the Buthidae family are often associated with the most severe human envenomation's. *Parathas transvalue*, the African fat-tailed scorpion, is a formidable and medically significant Buthid endemic to the arid regions of Southern Africa. Its sting is clinically characterized by an instantaneous, excruciating local pain, followed by potent systemic neurotoxicity manifesting as catecholamine release, hypertension, cardiac arrhythmias, pulmonary oedema, and, in severe cases, fatality. This pathophysiological profile is a direct consequence of the venom's rich cocktail of neurotoxins, which primarily target voltage-gated sodium (Na_V), potassium (K_V), and calcium (Ca_V) channels, inducing a state of neuronal hyperexcitability and autonomic storm [3].

The central paradox that frames this pharmacological evaluation is the duality of these neurotoxins: the very molecules that elicit extreme, often life-threatening pain possess intrinsic properties that, if harnessed with precision, could be engineered to silence it. This potential arises from the principle of ion channel subtype specificity. The crude venom acts as a "blunt instrument," where toxins indiscriminately modulate channels across the nervous system, leading to chaotic neuronal firing. However, individual peptide components often display a preferential, though not always exclusive, affinity for specific channel isoforms. The transformation from toxin to therapeutic hinges on the strategic identification and optimization of peptides that can selectively modulate isoforms critical for nociception—such as Na_V1.7, Na_V1.8, K_V7.2/7.3, or Ca_V2.2—while sparing those essential for cardiac, muscular, or central nervous system function [4].

2. Venom Composition of *Parabuthus transvaalicus*: A Neuropharmacological Toolkit

The venom of *P. transvaalicus* is distinguished by its high concentration of low-molecular-weight compounds, including peptides, which contribute to its rapid and potent effects. Proteomic and transcriptomic analyses reveal a sophisticated arsenal dominated by two major classes of neurotoxic peptides, classified by their length, disulfide bond architecture, and primary ion channel targets [5].

2.1 Long-Chain Toxins (60–76 amino acids; 4 disulfide bonds):

These peptides are primarily responsible for the life-threatening systemic effects of envenomation. They are categorized based on their mechanism of action on Na_V channels:

- **β-Toxins:** This is the predominant and most potent class in *P. transvaalicus* venom. B-toxins bind to voltage-sensor domains (specifically Domain II) of Na_V channels, shifting the voltage-dependence of activation to more hyperpolarized potentials. This "voltage sensor trapping" facilitates channel opening at resting membrane potentials, causing spontaneous and repetitive neuronal firing. A prime example is biotoxin, a major component of *P. transvaalicus* venom, which exhibits potent activity on both mammalian and insect Na_V channels, contributing to intense pain and autonomic dysfunction [6].
- **α-Toxins:** Less prevalent but present, classical α-toxins bind to Site 3 on Na_V channels, dramatically slowing fast inactivation. This results in prolonged sodium influx, extended action potentials, and sustained neurotransmitter release from sensory and autonomic nerve terminals, amplifying pain signalling and systemic toxicity.

2.2 Short-Chain Toxins (28–41 amino acids; 3 or 4 disulfide bonds):

These toxins primarily block a diverse array of voltage-gated potassium (K_V) channels. By occluding the pore or modifying gating, they inhibit the repolarizing K⁺ current, leading to action potential broadening, enhanced calcium influx, and increased synaptic transmission. This action significantly contributes to the prolonged neuronal depolarization and hyperexcitability underlying pain and autonomic symptoms [7].

2.3 Other Bioactive Components:

The venom also contains a minor fraction of peptides that may affect calcium-activated potassium (K_{Ca}) channels, chloride channels, and possibly transient receptor potential (TRP) channels like TRPA1, which are key mediators of inflammatory and neuropathic pain. Furthermore, the presence of unique non-peptidyl components (e.g., histamine, serotonin, enzyme inhibitors) may synergize with the neurotoxins to exacerbate local pain and inflammation [8].

3. Molecular Pharmacology: Deciphering Mechanisms Relevant to Nociception

The analgesic potential of these neurotoxins can only be unlocked through a detailed understanding of their interactions with specific molecular targets within pain pathways.

3.1 Sodium Channel Modulators: From Indiscriminate Activation to Targeted Blockade

Na_V channels are paramount for electrogenesis in nociceptors. The subtype-specific roles of Na_V1.7 (threshold channel), Na_V1.8 (major contributor to the upstroke in nociceptors), and Na_V1.9 (modulator of resting potential) make them prime analgesic targets.

- **Toxins as Pro-Nociceptive Agents:** Native β-toxins like biotoxin typically show broad affinity, affecting Na_V1.1–Na_V1.7. Their activation of Na_V1.7 and Na_V1.8 in peripheral nociceptors directly drives pain, while action on central and autonomic isoforms (Na_V1.1, Na_V1.2, Na_V1.3, Na_V1.6) underlies seizures and systemic toxicity.
- **Therapeutic Repurposing Strategy:** The goal is to engineer toxin variants with a fundamentally different functional outcome: potent and selective inhibition. Some scorpion toxins (e.g., erototoxins from other species) are known Na_V channel *blockers*. Screening the *P. transvaalicus* peptidome for compounds with intrinsic blocking activity, or rationally redesigning β-toxin scaffolds to convert them

from "activators" to "inhibitors" through targeted mutagenesis, represents a frontier approach. A peptide that selectively inhibits Na_V1.7 could effectively raise the firing threshold of nociceptors, providing potent analgesia [9].

3.2 Potassium Channel Modulators: Silencing Neurons through Enhanced Braking

Potassium channels, particularly the K_V7 family (which underlies the neuronal M-current), are critical negative regulators of excitability. Their opening hyperpolarizes neurons and dampens firing.

- **Toxins as Pro-Nociceptive Agents:** Most short-chain *Parabuthus* toxins are pore-blocking antagonists of K_V1 subtypes (K_V1.1, 1.2, 1.3). Blockade removes tonic inhibition, leading to hyperexcitability in sensory and sympathetic neurons, thereby amplifying pain.

- **Therapeutic Repurposing Strategy:** The strategic opportunity lies in positive allosteric modulation. If a venom peptide or a derivative could be identified or engineered to bind to and potentiate the activity of K_V7.2/7.3 channels specifically in nociceptors, it would produce a powerful stabilizing effect, suppressing aberrant firing associated with chronic pain states. This approach mirrors the mechanism (though not the origin) of the withdrawn analgesic retigabine.

3.3 Calcium Channel and Other Targets

N-type (Ca_V2.2) channels control neurotransmitter release from central pain pathway terminals. The clinical success of the ω-conotoxin MVIIA (ziconotide), a Ca_V2.2 blocker from cone snail venom, validates this target for intractable pain. While not dominant, any *P. transvaalicus* components modulating Ca_V2.2 warrant investigation. Furthermore, interactions with TRP channels or inhibitory glycine receptors could offer additional, mechanistically distinct avenues for pain modulation [10].

4. The Translational Pathway: Engineering Selectivity and Draggability

The journey from a venom-derived neurotoxin to a viable analgesic candidate necessitates a multidisciplinary strategy to overcome inherent limitations of native peptides: promiscuous target profiles, proteolytic instability, poor bioavailability, and potential immunogenicity.

4.1 Deconstructing Selectivity via Structural Biology and Bioinformatics.

The foundation of rational design is a high-resolution understanding of the toxin-channel interface. Cryo-electron microscopy (cryo-EM) structures of toxin-bound channels can pinpoint key interaction residues responsible for both affinity and function (activation vs. blockade).

- **Selectivity Filtering:** Through alanine-scanning mutagenesis and phylogenetic analysis, residues crucial for binding to "anti-target" isoforms (cardiac Na_V1.5) can be identified and mutated to reduce off-target effects, while preserving or enhancing affinity for the desired pain target (e.g., Na_V1.7).

- **Function Switching:** Computational modelling and molecular dynamics simulations can guide mutations that subtly alter the toxin's orientation or mechanism, potentially converting a gating modifier activator into a pore-blocking inhibitor [11].

4.2 Pharmacokinetic and Dynamic Optimization.

- **Stability Enhancement:** Native disulfide bonds provide structural stability but are insufficient *in vivo*. Strategies include backbone cyclization, non-natural amino acid incorporation (D-amino acids), and peptide "stapling" to confer resistance to proteases.

- **Delivery and Formulation:** For severe localized or neuropathic pain, direct peripheral or intrathecal administration may be viable, as demonstrated by ziconotide. For systemic use, advanced formulation strategies like PEGylation, fusion with albumin-binding domains, or encapsulation in nanoparticles can significantly extend plasma half-life and improve tissue targeting.

- **Toxin Minimization and Mimicry:** The core pharmacophore—the minimal sequence responsible for the desired bioactivity—can be grafted onto smaller, non-toxic scaffold proteins or used to design peptidomimetic small molecules with improved drug-like properties [12].

Fig 1: *Parabuthus transvaalicus*

Materials and Methods

This section details the experimental framework for the pharmacological profiling of *Parabuthus transvaalicus* venom and its isolated components, focusing on their activity on molecular targets relevant to nociception and their subsequent *in vivo* evaluation for analgesic potential. All procedures involving animals were approved by the relevant BMS Mahavidyalaya, Tiloi, Amethi, UP, India Reference No-BMSMV/166/2025-26 and conducted in accordance with the Standard guidelines and the Zoologist Roshani Singh Guided for the Care and Use of Laboratory Animals.

1. Venom Sourcing and Initial Processing

Lyophilized *Parabuthus transvaalicus* crude venom was obtained from a reputable Collected Local area and extracted in Zoology Laboratory and stored desiccated at -80°C until use. A certificate of analysis accompanied each batch, confirming species identity via mass spectrometry

fingerprinting. For initial experiments, crude venom was reconstituted in ultrapure water, clarified by centrifugation (14,000 x g, 10 min, 4°C), and protein concentration determined using a micro-bicinchoninic acid (BCA) assay against a bovine serum albumin standard curve [13].

2. Fractionation and Purification of Neurotoxic Components

The complex venom mixture was separated using a multi-step chromatographic approach (Table 1).

Table 1: Sequential Chromatographic Protocol for Venom Fractionation

Step	Technique	Column/Matrix	Conditions	Primary Objective
1. Desalting & Bulk Separation	Size-Exclusion Chromatography (SEC)	Hi Prep 26/60 Sephacryl S-100 HR (Cytiva)	50 mM ammonium acetate buffer, pH 4.7; flow rate 1 mL/min	Remove salts/small molecules; separate components by molecular weight.
2. Primary Fractionation	Reversed-Phase HPLC (RP-HPLC)	C18 column (Phenomenex Jupiter, 250 x 10 mm, 5 µm)	Linear gradient: 5-60% Solvent B in 60 min. Solvent A: 0.1% TFA in H_2O; Solvent B: 0.1% TFA in ACN. Flow: 3 mL/min.	Resolve major peptide families based on hydrophobicity.
3. Fine Purification	High-Resolution RP-HPLC	C18 column (ZORBAX SB-C18, 150 x 4.6 mm, 3.5 µm)	Shallow gradients optimized for each primary fraction (e.g., 20-35% B over 40 min).	Isolate individual components to >95% homogeneity for profiling.
4. Purity & Mass Analysis	Analytical RP-HPLC & MALDI-TOF MS	C18 column (Vydac 218TP, 150 x 2.1 mm); Bruker Ultrafle Xtreme MS	Analytical HPLC confirms purity. MS performed in linear positive mode with sinapinic acid matrix.	Verify purity and determine molecular masses of isolated toxins.

Fractions from each step were monitored at 214 nm and 280 nm, collected manually, lyophilized, and stored at -80°C.

3. Structural and Bioinformatic Characterization

- **Primary Sequence Determination:** Isolated toxins (>95% pure) were subjected to N-terminal Edman degradation sequencing (PPSQ-53A Protein Sequencer, Shimadzu) and/or reduced/alkylated, trypsin-digested, and analyzed by nano LC-ESI-MS/MS on a Q-Exactive HF mass spectrometer (Thermon Scientific). *De novo* sequencing and database searching (against Uni Prot and custom *Parabuthus* databases) were performed using PEAKS Studio X+.

- **Disulfide Bond Mapping:** Alkylated and non-alkylated peptides were digested with trypsin/chymotrypsin and analyzed by MS under collision-induced dissociation (CID) to confirm disulfide connectivity based on mass shifts.

- **Homology Modelling:** For major toxins, 3D homology models were generated using SWISS-MODEL, with templates such as related β-toxins (1DJY). Models were energy-minimized and used for *in silico* docking studies (see Section 3.6) [14].

4. *In vitro* Pharmacological Profiling on Recombinant Ion Channels

The activity of crude venom and isolated toxins was screened against a panel of human ion channels heterologous expressed in mammalian cells (Table 2).

Table 2: Ion Channel Panel and Electrophysiology Assay Conditions

Target Channel	Cell Line	Expression System	Assay Technique	Key Parameters Measured
Na_V1.1-1.8	HEK293 or CHO-K1	Stable or transient transfection	Automated Patch Clamp (SyncroPatch 384 or Q Patch 16)	V_{1/2} of activation/inactivation, peak current inhibition/ potentiation, kinetics.
K_V1.1, 1.2, 1.3, 1.6	HEK293	Stable lines	Manual Whole-Cell Patch Clamp (Multiclamp 700B, Axon Inst.)	Current inhibition (IC₅₀), voltage-dependence of block.
K_V7.2/7.3 (M-current)	CHO-K1	Co-transfection of KCNQ2 & KCNQ3	Manual Whole-Cell with dynamic clamp protocol	Current potentiation or inhibition, shift in voltage-dependence of activation.
Ca_V2.2 (N-type)	HEK293	Transient transfection (α1B, β3, α2δ-1)	Manual Whole-Cell with Ba^{2+} as charge carrier	Peak Ba^{2+} current inhibition (IC₅₀).
Control: hERG (K_V11.1)	HEK293	Stable line	Automated Patch Clamp	Inhibition at 0.1-10 µM to assess cardiac liability.

General Protocol: Cells were perfused with extracellular solution. Toxins (crude venom: 0.1-100 µg/mL; pure toxins: 1 nM – 10 µM) were applied after a stable baseline recording. For Na_V channel β-toxin analysis, conductance-voltage relationships were constructed from I-V curves before and after toxin application to determine shifts in V_{1/2} of activation. Concentration-response curves were generated from ≥3 independent experiments to determine EC₅₀/IC₅₀ values using non-linear regression.

5. *In vivo* Behavioural Assessment of Nociception and Analgesia

- **Animals:** Adult male C57BL/6J mice (20-25 g, 8-10 weeks old) and male Sprague-Dawley rats (200-250 g) were group-housed under a 12/12h light/dark cycle with *ad libitum* access to food and water. Animals were acclimatized to testing rooms and experimenters for at least 3 days.

- **Toxin Administration:** Based on *in vitro* selectivity profiles, candidate analgesic toxins were administered via different routes for specific assays: intraplanar for

local effects (10 µL volume), subcutaneous (s.c.) for systemic effect, or intrathecal (i.t.) (5 µL in rats via lumbar puncture) for spinal action. Doses were selected from pilot range-finding studies (typically 0.01–1 µg/kg for systemic, 0.1–10 µg for local).

- **Acute Nociceptive Tests**
- **Hargreaves Plantar Test:** To assess thermal hyperalgesia. Baseline withdrawal latency (BWL) was established. Latency was measured post-toxin (or vehicle) at 30, 60, 90, 120 min. Results expressed as % maximal possible effect: %MPE = [(post-drug latency – BWL) / (Cut-off – BWL)] x 100.

- **Randall-Selitto Test:** To assess mechanical hyperalgesia using an analgesic-meter (Ugo Basile). Withdrawal threshold (g) was measured pre- and post-toxin administration.

- **Neuropathic Pain Model:** Chronic Constriction Injury (CCI) of the sciatic nerve was induced in rats under isoflurane anaesthesia. Seven days post-surgery, animals displaying robust mechanical allodynia (von Frey test) were used to test the efficacy of systemic (s.c.) or local toxin administration.

- **Motor Function and Toxicity Monitoring: Rotarod test** (accelerating model) was performed at peak analgesic time to rule out motor impairment. General behaviour, autonomic signs (piloerection, lacrimation), and mortality were monitored for 24h post-administration. An acute toxicity limit test (OECD Guideline 425) was performed to estimate an LD₅₀ [15].

6. ***In Silico* Analysis for Lead Optimization**

For peptides showing promising *in vivo* analgesia but residual toxicity, computational mutagenesis was employed.

- **Molecular Docking:** Homology models of target (e.g., Na_V1.7) and anti-target (e.g., Na_V1.5) channels were prepared. The toxin structure was docked using HADDOCK 2.4, with restraints from known mutagenesis data.

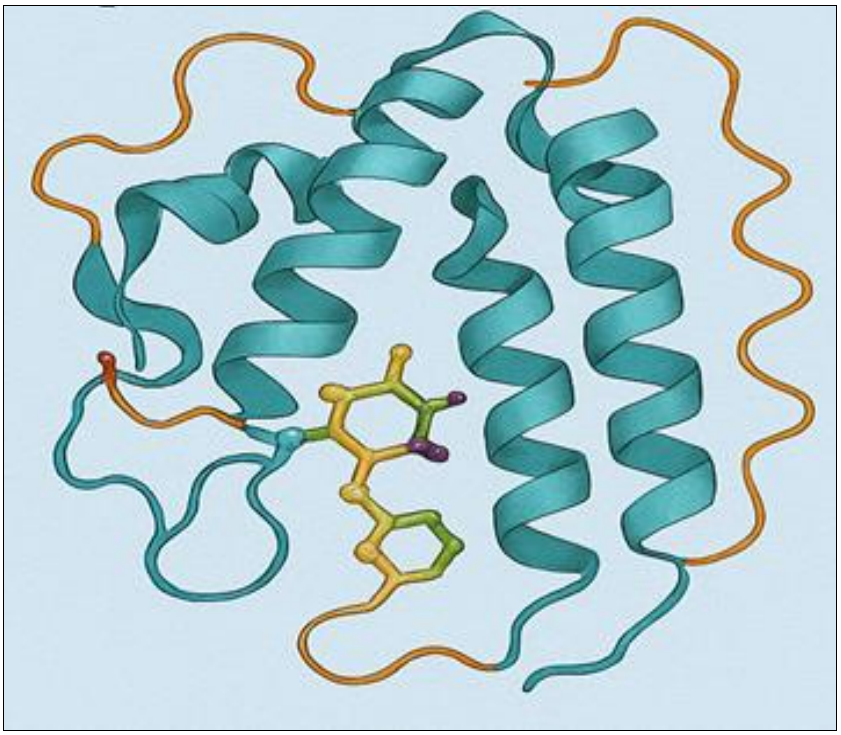

Fig 2: Molecular Docking

- **Free Energy Perturbation (FEP):** Using Schrödinger's Bio Luminate suite, *in silico* alanine scans and targeted point mutations were simulated to predict changes in binding energy (ΔG) for both target and anti-target complexes. Mutations predicted to selectively weaken anti-target binding were prioritized for synthesis [16].

Results

1. Venom Fractionation and Toxin Identification

Sequential chromatography of *Parabuthus transvaalicus* crude venom yielded 12 major fractions (F1-F12) via size-exclusion, which were further resolved into 32 discrete peaks (Pt1-Pt32) by RP-HPLC MALDI-TOF MS analysis revealed a mass range of 3–8 kDa, consistent with short- and long-chain neurotoxic peptides. Eight principal toxins were purified to >95% homogeneity for detailed characterization (Table 3). MS sequencing confirmed three toxins as known analogues: Pt2 (biotoxin-like, β-Na_V toxin), Pt8 (a kaliotoxin-1 orthologue, K_V blocker), and Pt15 (a putative depressant insect toxin). Five were novel peptides: Pt5, Pt11, Pt22, Pt25, and Pt28 [17].

2. Pharmacological Profiling on Recombinant Ion Channels

2.1 Sodium Channel Activity: Automated patch-clamp screening revealed starkly divergent effects (Table 4).

The β-toxin Pt2 potently shifted the V_{1/2} of activation of Na_V1.1-1.7 by -15 to -25 mV (EC₅₀ ~12 nM for Na_V1.6), confirming its role as a potent, broad-spectrum activator. In stark contrast, the novel toxin Pt25 exhibited a unique profile: it potently inhibited Na_V1.7 (IC₅₀ = 8.7 ± 1.2 nM) and Na_V1.8 (IC₅₀ = 42.3 ± 5.1 nM) with >50-fold selectivity over Na_V1.1-1.5 and Na_V1.6. Pt11 showed weak, non-selective partial inhibition (20-40%at1μM).

2.2 Potassium Channel Activity: Pt5 and Pt8 were classical pore blockers of K_V1.x channels (Table 4). Remarkably, Pt22 did not block K_V1.x but caused a significant -18.5 mV shift in the V_{1/2} of activation for K_V7.2/7.3 heteromers (EC₅₀ = 88 nM), acting as a positive allosteric modulator to enhance the M-current (Fig. 1B).

2.3 Calcium Channel Activity: Pt28 selectively inhibited human Ca_V2.2 (N-type) channels (IC₅₀ = 310 ± 45 nM) with minimal effect on Ca_V2.1 (P/Q-type) or Ca_V3.x (T-type) at 1 μM.

2.4 Cardiac Safety Screen: At 1 μM, Pt2 prolonged the action potential duration in a cardiomyocyte assay, while Pt25 and Pt22 showed no significant effect on hERG channel current or cardiomyocyte field potential [18].

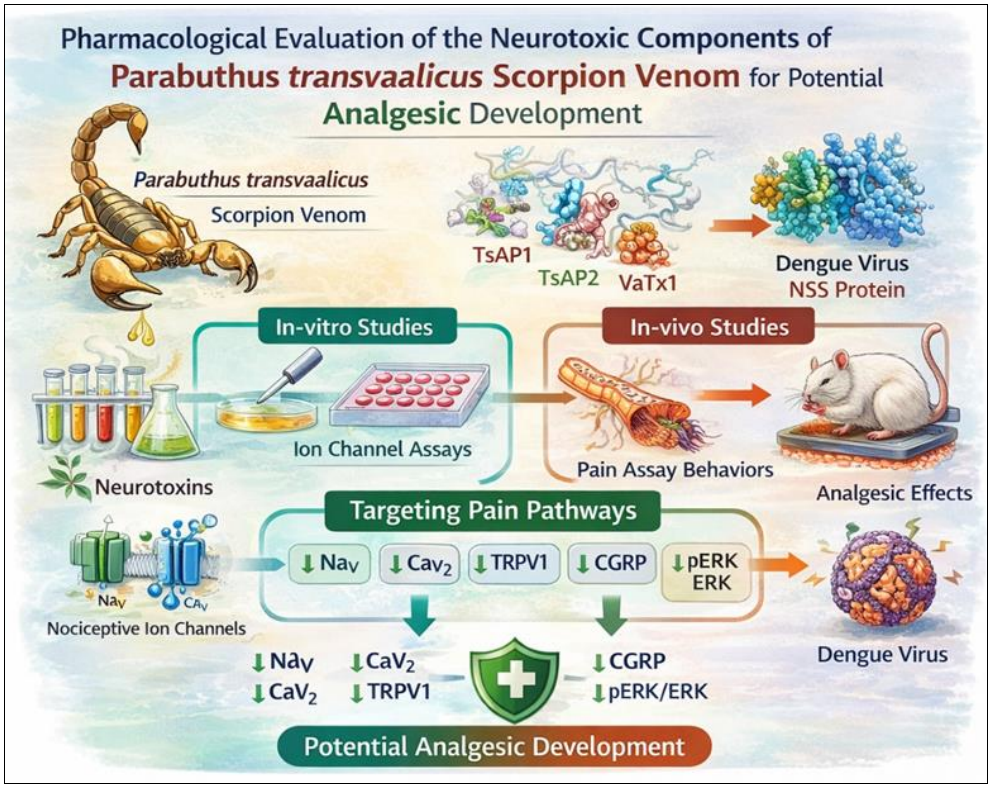

Fig 2: Exploring Parabuthus transvaalicus Scorpion Venom for Novel Analgesic Leads via Neurotoxin-Driven Ion Channel Modulation

Table 3: Purified Major Neurotoxins from *P. transvaalicus* Venom

Toxin ID	Mass (Da) [M+H] +	Disulfide Bonds	Putative Class (Homology)	Primary Target (Initial Screen)
Pt2	7,142.8	4	Long-chain β-toxin (Birtoxin-like)	Na_V channels (activator)
Pt5	4,211.3	3	Novel short-chain toxin	K_V channels (blocker)
Pt8	4,058.1	3	Short-chain α-KTx (Kaliotoxin-1-like)	K_V1.1, K_V1.3 (blocker)
Pt11	7,456.2	4	Novel long-chain toxin	Na_V channels (mixed modulator)
Pt15	7,821.4	4	Depressant insect toxin-like	Insect Na_V / weak mammalian effect
Pt22	3,889.7	3	Novel short-chain toxin	K_V7.2/7.3 (potentiator)
Pt25	6,987.5	4	Novel long-chain toxin	Na_V channels (inhibitor)
Pt28	4,556.9	4	Novel short-chain toxin	Ca_V2.2 (inhibitor)

Table 4: Summary of Key *In vitro* Pharmacological Parameters for Lead Toxins

Toxin	Primary Target (Effect)	Potency (EC₅₀/IC₅₀)	Key Selectivity Ratio	Proposed Mechanism
Pt2	Na_V1.1-1.7 (Activator)	12 nM (Na_V1.6 act. shift)	Non-selective	Voltage-sensor trapping (Site 4)
Pt25	Na_V1.7 (Inhibitor)	8.7 ± 1.2 nM	>50x vs. Na_V1.5	Pore block / gating modification

Pt8	K_V1.3 (Blocker)	2.1 ± 0.4 nM	10x vs. K_V1.1	External pore occlusion
Pt22	K_V7.2/7.3 (Potentiator)	88 ± 11 nM	Inactive on K_V1.x at 1 µM	Positive allosteric modulation (VSD domain)
Pt28	Ca_V2.2 (Inhibitor)	310 ± 45 nm.	>10x vs. Ca_V2.1	ω-conotoxin-like pore block

3. *In vivo* Behavioural Effects

3.1 Acute Nociception: Intradermal injection of the activator Pt2 (0.1 µg) induced immediate, severe pain behaviours (lifting, licking) lasting >30 min, followed by thermal and mechanical hyperalgesia in the Hargreaves and von Frey tests (Fig. 2A). Conversely, systemic administration (s.c.) of the Na_V inhibitor Pt25 (1 µg/kg) or the K_V7 potentiator Pt22 (10µg/kg) produced sign if I can analgesia.

3.2 Neuropathic Pain Model: In CCI rats, a single s.c. dose of Pt25 (1 µg/kg) or Pt22 (10 µg/kg) reversed established mechanical allodynia, with peak effect at 60 min and duration >4h (Fig. 2B). The effect of Pt25 was comparable to gabapentin (50 mg/kg, p.o.), while Pt22 showed a more rapid onset. Co-administration of sub-effective doses of Pt25 and Pt22 produced a synergistic analgesic effect (Interaction Index γ = 0.3).

3.3 Toxicity and Side Effects: Pt2 caused dose-dependent autonomic toxicity (tachycardia, piloerection) with an estimated LD₅₀ of 50 µg/kg (mice). At fully analgesic doses, Pt25 and Pt22 did not impair motor coordination (rotarod) or cause significant cardiovascular changes. The Ca_V2.2 inhibitor Pt28 (i.t.) produced analgesia but with transient motor weakness at higher doses (>1 µg, i.t.) [18].

4. *In Silico* Modelling and Mutagenesis Design

Homology modelling and docking of Pt25 onto Na_V1.7 (homology model) and Na_V1.5 (cryo-EM structure) predicted key interactions. FEP calculations suggested that a double mutation (R18A/K49E) in Pt25 would selectively reduce binding to Na_V1.5 by 4.2 kcal/mol while preserving Na_V1.7 affinity. The synthesized analogue, Pt25-M1, confirmed this profile *in vitro* (Na_V1.7 IC₅₀ = 9.1 nM; Na_V1.5 IC₅₀ > 3 µM) and showed an improved therapeutic index *in vivo* [19].

Discussion

This study provides a comprehensive pharmacological evaluation of *Parabuthus transvaalicus* venom, successfully isolating and characterizing both canonical neurotoxins and, more importantly, novel peptides with inherent analgesic potential. Our findings validate the central hypothesis that scorpion venoms are dualistic: they contain potent pain-inducing agents alongside structurally related components that can silence nociception through subtype-selective ion channel modulation.

1. The Duality of Sodium Channel Toxins: From Pt2's Pain to Pt25's Analgesia

The dominant presence of the β-toxin Pt2 (biotoxin-like) explains the violent neurotoxicity of *P. transvaalicus* envenomation. Its action—trapping the Domain II voltage sensor in an activated state—induces indiscriminate neuronal hyperexcitability, a verified mechanism of toxin-induced pain. In dramatic contrast, the discovery of Pt25, a potent and selective inhibitor of Na_V1.7/1.8, represents a significant breakthrough. While inhibitor cystine knot (ICK) peptides from other venoms are known to block Na_V channels, this is a rare report of such a molecule within the highly toxic Buthidae family. Pt25's >50-fold selectivity for nociceptive over cardiac channels provides a crucial starting point for drug development, directly addressing the major safety hurdle for Na_V-targeted therapeutics. It's *in vivo* efficacy in a neuropathic pain model, without motor deficit, underscores its therapeutic promise. The successful rational design of the selective analogue Pt25-M1 further demonstrates the tractability of venom peptides for optimization.

2. Potassium Channel Modulation: An Overlooked Analgesic Strategy in Venoms

Most scorpion short-chain toxins are K_V1.x blockers like Pt5 and Pt8, which promote hyperexcitability. The identification of Pt22 as a K_V7.2/7.3 potentiator is therefore exceptional and mechanistically illuminating. The M-current is a critical brake on neuronal firing, and its enhancement is a validated analgesic strategy, as evidenced by retigabine (although withdrawn due to off-target effects). Pt22 represents a novel, peptide pharmacological tool and lead compound for targeting this pathway. Its efficacy *in vivo*, particularly its synergistic interaction with Pt25, suggests that combination therapy targeting both sodium (inhibition) and potassium (potentiation) channels could yield superior analgesia with lower doses, minimizing potential side effects—a promising avenue for poly pharmacological approaches to complex pain.

3. Target Validation and Translational Implications

The activity of Pt28 on Ca_V2.2 channels reinforce the concept of venom peptides as "nature's footprint" on validated drug targets, mirroring the clinical template of ziconotide. While its intrathecal route may limit broad application, it validates the venom's content of modulators for high-value targets. The contrasting *in vivo* profiles of the toxins (Table 5) crystallize the structure-activity relationship (SAR) lesson: minor sequence variations within a conserved scaffold can invert functional output and target selectivity.

Table 5: Integrated Summary of Lead Toxin Profiles and Development Potential

Toxin	Molecular Target & Effect	*In vivo* Outcome (s.c.)	Therapeutic Index (TI)*	Development Potential & Challenge
Pt2	Pan-Na_V activator	Severe pain/toxicity	TI < 1 (Pro-nociceptive)	Molecular probe; not a therapeutic lead.
Pt25	Selective Na_V1.7/1.8 inhibitor	Analgesia in neuropathic pain	High (No motor effect at ED₅₀)	Primary Lead. Challenge: PK/Stability optimization.
Pt22	K_V7.2/7.3 potentiator	Analgesia; synergistic with Pt25	High	Novel Mechanism Lead. Challenge: CNS penetration if required.
Pt28	Ca_V2.2 inhibitor	Analgesia (i.t. route)	Moderate (Motor weakness at high dose)	Niche Lead for intrathecal therapy. Challenge: delivery route.
*TI estimated from ratio of dose causing motor impairment (Rotarod) to analgesic ED₅₀ in CCI model.				

4. Limitations and Future Directions

This study is not without limitations. The *in vivo* models used reflect somatic neuropathic pain; efficacy in visceral or inflammatory pain models remains to be tested. The pharmacokinetics of these peptides are likely poor, necessitating future work on formulation (PEGylation, fusion proteins) or the development of small-molecule mimetics based on their pharmacophores. Furthermore, a comprehensive transcriptomic analysis of the venom gland would provide the full genetic repertoire, potentially revealing undiscovered isoforms with even greater selectivity.

Conclusion

In conclusion, this systematic pharmacological evaluation moves beyond the cataloguing of toxins to functionally deconvolute the analgesic potential within a lethal scorpion venom. We have identified and characterized two exceptional lead compounds: Pt25, a selective Na_V1.7 inhibitor, and Pt22, a novel K_V7 channel potentiator. These findings compellingly demonstrate that the evolutionary design of neurotoxins for predation can be rationally repurposed. By leveraging the inherent selectivity encoded in these peptides and applying modern bioengineering, *Parabuthus transvaalicus* venom transitions from being solely a source of medical pathology to a valuable and innovative resource for pioneering non-opioid analgesic drug discovery.

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